

Document Control Number <b>WI-B-T-1-6</b>	<b>WORK INSTRUCTION</b> USDA, APHIS, PPQ, CPHST, National Plant Germplasm and Quarantine Laboratory, Bldg 580, BARC-East, Beltsville, MD 20705	Revision Number <b>4</b>
Effective Date: <b>April 3, 2006</b>	Quantitative Multiplex Real-Time PCR (qPCR) for Detection of <i>Phytophthora ramorum</i> Using a TaqMan System on the Cepheid SmartCycler® and the ABI 7900/7000	<b>Page 1 of 11</b>

## INTRODUCTION

This real-time or quantitative PCR (qPCR), originally developed by the Central Science Laboratory (CSL), York, UK, was validated by USDA-APHIS-PPQ-CPHST for providing diagnostic determinations for the *Phytophthora ramorum* emergency program. The result was that minor modifications were made for optimization of the assay on a Cepheid SmartCycler® II. There were a few chemical and physical parameter changes from the original CSL method, however, the primers and probe for *P. ramorum* and the COX internal control primers and probe are the same. The *P. ramorum* (Pram) qPCR can detect *P. ramorum* in a broad range of host plants. To date, DNA isolated from plant samples using the Qiagen DNeasy® Plant Mini Kit and tested with this protocol have shown little to no interference due to plant inhibitors. Using Qiagen's DNeasy Mini Plant DNA extraction kit, the range of detection (the interval between the upper and the lower concentration of the pathogen in environmental samples from which the agent can be reliably and reproducibly detected) of this protocol is from 200 ng to 2 fg of genomic DNA. The test utilizes internal control primers and probe based on the COX gene that are multiplexed with *P. ramorum* primers and probe for each DNA sample in order to confirm that amplifiable DNA is present in environmental samples that test PCR-negative for *P. ramorum*. The qPCR protocol can also be used for on-site detection of *P. ramorum* in the Cepheid system.

The protocol listed in this work instruction was evaluated in a ring-test involving several laboratories with consistent results. High concentration of genomic DNAs generated from isolated cultures exhibited cross amplification with isolates from azalea and with *P. lateralis*. Environmental DNA samples showed late cycle cross amplification with crossing thresholds (Cts) of 38 and higher, and only a few Ct readings of approximately 35. During the ring-test on the Cepheid SmartCycler® platform some of the participating laboratories were asked to extend the protocol to two models of the ABI system (models 7900 and 7000) and successful results were achieved eliminating the cross reactivity of the assay. Included in this work instruction are the parameters for the ABI 7900 that were determined by the USDA-AMS Laboratory in Gastonia, NC and were additionally verified successfully on the ABI 7000 by the University of Tennessee.

Each diagnostic run of the *P. ramorum* qPCR must include at least one positive control and one negative control (negative template control containing either TE buffer or H<sub>2</sub>O). If the positive control is *P. ramorum* genomic DNA, a

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dilution series and qPCR will be needed to determine a working dilution/concentration for subsequent PCR assays. If possible, an environmental sample known to be positive for *P. ramorum* should be included.

## **A. Materials and Equipment:**

### **A.1 Equipment needed:**

Cepheid SmartCycler® II (version 2)  
SmartCycler® Tubes-25µl (Part # 900-0003, Cepheid)  
Dedicated, calibrated pipettors (P10, P50, P200)  
Sterile filter (barrier) pipette tips (P10, P50, P200)  
Sterile filter (barrier) ART® gel loading pipette tips (P-100, Part # 2155, Molecular BioProducts)  
Amber-colored 1.5 ml Flextubes (Part # 22-36-351-4, Eppendorf)  
0.6 ml microcentrifuge tubes (pre-sterilized, certified DNase & RNase free, any vendor)

### **A.2 Materials needed:**

Platinum Taq DNA Polymerase (Cat.# 10966-034, Invitrogen)  
dNTP MIX (10 mM solution, PCR reagent) (Product # D 7295, Sigma)  
Molecular Biology Grade Water (# 955-15-503-3, Eppendorf)  
D-(+)-Trehalose dehydrate (T9531-10G, Sigma).  
2X Taqman Master Mix (ABI Taqman Universal PCR Master Mix Part #4304437)  
*Note: Cepheid Omnimix beads are not to be used with this protocol.*

## **B. The PCR Reaction**

### **B.1 TaqMan primers and probes**

**Note:** Primers and probes were synthesized by Integrated DNA Technologies [IDT]  
[www.IDTDNA.com](http://www.IDTDNA.com)

<b>Primer Name</b>	<b>Primer Sequence</b> (synthesized by Integrated DNA Technologies, Inc.)
	(Purification - Standard Desalting)
Pram-114F	5' – TCA TGG CGA GCG CTT GA -3'
Pram-1527-190R	5' – AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT – 3'
COX-F	5' – CGT CGC ATT CCA GAT TAT CCA – 3'
COX-RW	5' – CAA CTA CGG ATA TAT AAG RRC CRR AAC TG – 3'
<b>Probe Name</b>	<b>Probe Sequence</b> (synthesized by Integrated DNA Technologies, Inc.)
	(Purification - Dual HPLC purification)
Pram1527-134T	5' - /56-FAM/TTC GGG TCT GAG CTA GTA G/3BHQ_1/ - 3'
COX-P	5' - /5TexRd-XN/AGG GCA TTC CAT CCA GCG TAA GCA /3BHQ_2/ - 3'

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## B.2 Reagent Preparation:

### 25% Trehalose:

2.5 grams of trehalose is brought up to 10 ml with molecular biology grade water, stirred to dissolve and 1 ml aliquots are stored at -20°C.

### Freezer stock solutions (100 µM ) of the probes and primers, and working stock dilutions:

Dissolve primers and probes in molecular biology grade H<sub>2</sub>O or TE buffer and store at -20°C. Probes should be aliquotted in small amounts suitable for one-day of qPCR runs (depending the PCR levels in each lab, for example 20 µl) in amber microfuge tubes to protect the fluorescent labels from degrading due to light exposure.

### Working solutions of primers and probes:

- Prepare 2 µM working solutions of each of the two PCR primer pair mixtures one for the SOD primer pair (Pram114F/Pram1527-190R) and one for the COX primer pair (COX-F/RW). Once diluted, the final concentration of each primer in the mix is 2 µM. For example, to make 1 ml of a 2 µM mix of primers Pram114F/Pram1527-190R, add 20 µl of the 100 µM Pram114 primer stock and 20 µl of the 100 µM Pram1527-190R primer stock to a 1.5 ml microfuge tube and bring up to 1 ml with 960 µl of sterile molecular grade water. Store 100 µl aliquots at -20°C.
- Prepare 2 µM solutions of each probe in molecular biology grade H<sub>2</sub>O or TE buffer. Store small aliquots (20 µl) of probes at -20°C in 1.5 ml amber-colored microfuge tubes to protect the primer from light exposure. Small aliquots of probe are desirable since probes are sensitive to frequent freeze/thaw cycles. Prepare the probe dilutions by diluting the 100 µM probe stock solution.

## B.2 qPCR Settings and Reaction Parameters:

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#### **B.2.a. Cepheid SmartCycler® Set-up:**

Verify the following settings on the SmartCycler® II prior to use:

- Stage 1: Hold at 95°C for 20 seconds with optics off.
- Stage 2: repeat 46 times and 2-Temperature Cycle.
  - the first temperature cycle, set 95°C for 1 second with optics off
  - the second temperature cycle, set 58°C for 40 seconds with optics on.

Use a temperature ramping rate of 3.0 degrees per second.

#### **B.2.b. ABI 7900 and 7000 System Settings:**

Verify the following setting on the ABI 7900 and 7000 systems:

- a. Stage 1: Hold at 50 °C for 2 minutes
- b. Stage 2: Hold at 95 °C for 10 minutes
- c. Stage 3: Repeat 46 times
  1. First Temperature Cycle, 95 °C for 15 seconds
  2. Second Temperature Cycle, 58 °C for 15 seconds
  3. Third Temperature Cycle, 72 °C for 30 seconds

Use a ramping rate of 3.5 degrees per second.

**Note:** To reduce the potential of false negative results the *P. ramorum* qPCR method cycle length parameter was adjusted to 46 cycles. The validated *P. ramorum* qPCR method was compared to the existing nested *P. ramorum* PCR procedure on over 800 environmental DNA samples during the second phase of the validation procedure. Analysis of this data indicates that there are a very small percentage of environmental DNA samples with Ct values between 40 and 46 that test positive in the nested PCR procedure.

#### **B.2.c. Reagent parameters for (i) the Cepheid SmartCycler® or (ii) the ABI 7900 or 7000:**

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**i. Standard Master Mix Preparation for the Cepheid SmartCycler®:**  
Prepare the standard master mix as described in Table 1.

**Table 1: Standard Master Mix for qPCR for the Identification of *P. ramorum* using the Cepheid SmartCycler®.**

Reagents	Volume (µl) for each reaction	Volume (µl) 16 reactions	Volume (µl) 17 reactions (16 + 1 rxn)*	Final concentration
10X PCR Buffer**	2.5	40.0	42.5	1X
H <sub>2</sub> O (sterile molecular grade)	2.7	43.2	45.9	
Trehalose (25%)	5.0	80.0	85.0	5%
MgCl <sub>2</sub> (50 mM)	3.0	48.0	51.0	6.0 mM
Primers Pram 114F/1527-190R (2 µM mix)	3.0	48.0	51.0	240 nM
Probe Pram 1527-134-T (2 µM)	1.5	24.0	25.5	120 nM
Primers COX-F/RW (2 µM mix)	3.0	48.0	51.0	240 nM
Probe COX (2 µM)	1.5	24.0	25.5	120 nM
dNTPs (10 mM each)	0.6	9.6	10.2	240 nM
Platinum Taq (5U/µl)	0.2	3.2	3.4	1 U
Total	23.0	368.0	391.0	N/A

\* Use this formula to ensure enough volume is made for all 16 reactions.

\*\*Use 10X buffer supplied with Platinum Taq.

**ii. Standard Master Mix Preparation for the ABI 7900 or 7000:**  
Prepare the standard master mix as described in Table 2.

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**Table 2: Standard Master Mix for qPCR for the Identification of *P. ramorum* using the ABI 7900 or 7000.**

Reagents	Volume (µl) for each reaction	For 100* reactions (96+4) (µl)	Final concentration
2X Taqman Master Mix (w/UNG)	12.5	1,250.0	1X
Pram Primer 114F (2 µM)	0.625	62.5	50 nM
Pram Primer 1527-190R (2 µM)	2.5	250.0	200 nM
Probe Pram 1527-134-T (2 µM)	1.25	125.0	100 nM
Primer COX-F (2 µM)	1.25	125.0	100 nM
Primer COX-RW (2 µM)	1.25	125.0	100 nM
Probe COX-P (2 µM)	1.25	125.0	100nM
Trehalose (25%)	2.375	237.50	5%
Total	23.0	2,300.0	

\*Four additional reactions were added to have extra master mix reagents so there is adequate volume for the 96 PCR reactions.

### **B.3 The qPCR assay:**

1. Pipette 23 µl master mix into 0.6 ml microfuge tubes, or into the 96-well PCR plate for the ABI system.
2. Add 2 µl of sample (environmental) DNA. DNA from environmental (test) samples must be tested both undiluted and as a 1:10 dilution in sterile molecular grade H<sub>2</sub>O. [For control reactions add 2 µl of positive control DNA or negative template control (H<sub>2</sub>O or TE buffer only) as required to verify a valid PCR run.]

The following steps are for the Cepheid SmartCycler®:

3. Pipette up and down to thoroughly mix then transfer 25 µl into the 25 µl Cepheid SmartCycler® tubes using barrier/filter gel loading pipette tips.
4. Spin the Cepheid tubes in the centrifuge provided from Cepheid for 10 sec. Make sure no bubbles remain on the sides of the flat diamond-shaped area of the Cepheid tube. Place the tubes in the SmartCycler® in the appropriate I-core modules.
5. Turn on the SmartCycler® Block first, followed by the SmartCycler® software. If not done in this order the machine will give an error message.

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6. To begin amplification: (instructions contained in the SmartCycler® manual)
  - i. Select “Create Run”
  - ii. Give the run a name (i.e.: date and protocol)
  - iii. Select dye set (for this qPCR it is “FTTC25”)
  - iv. Select “add/Remove Sites” to select the protocol
  - v. Select the sites with the samples and click on ► symbol to add sites to the right column, and
  - vi. Select “Start Run” found in the lower left-hand corner of the screen.

(Steps i. through v. can be completed prior to master mix set-up).

### **C. Assessment of the *P. ramorum* qPCR: Using the SmartCycler® software Version 2**

Analyze data from the run as per the thermocycler Manufacturer’s instructions. For the sake of discussion in sections C.1 to C.3 assume that FAM is the *P. ramorum* probe fluorescent label and that Texas Red (TxR) is the COX internal control probe fluorescent label. (This is an example for the Cepheid SmartCycler® system so insert the JOE or VIC-labeled COX control in this explanation if the ABI system is used.)

#### **C.1. Assessment of *P. ramorum* (FAM dye) Cts:**

If the positive control sample DNA (environmental or pure culture DNA) tests negative (i.e.: FAM Ct = 0 or very low Ct ≤ 13, or very high Ct 38-46), or your negative control (H<sub>2</sub>O or TE buffer only) tests positive (i.e.: FAM Ct > 0) the entire run is invalid. No sample results may be used and all samples must be retested.

Positive controls from the same genomic DNA isolation (from *P. ramorum* culture) should give a similar FAM Ct when used at the same dilution factor in subsequent PCR reactions. For example, a 1:100 dilution used at 2 µl in runs 1, 5, and 10 should have FAM Cts that are very close, ± 1 to 3 Cts. If this is not the case and, for example, the Cts vary by more than 4, it may indicate that either the control is going bad or the PCR reaction did not work as expected.



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A positive control (environmental sample or genomic DNA) that tests negative should be retested at a dilution of 1:100 to determine if the PCR was inhibited because the positive control DNA was highly concentrated.

### **C.2. Assessment of COX Internal Control (TxR dye) Cts:**

The COX primers target plant DNA sequences only and will not amplify *Phytophthora* or *P. ramorum* genomic DNA, including positive controls from genomic or culture DNA. The COX internal control reaction in an environmental sample will typically exhibit Cts in the low 20s to middle 30s when amplifiable DNA is present.

If an environmental positive control DNA has a COX internal control Ct = 0, or very low Ct  $\leq 13$ , or very high Ct = 36-46, then the entire run is invalid. No sample results obtained in the run may be used and all samples must be retested.

If an environmental sample has a COX internal control Ct = 0, or very low Ct  $\leq 13$ , or very high Ct = 36-46, then that specific sample may need to be retested. NOTE: Observations made by NPGBL and some field laboratories indicate that some environmental samples positive for *P. ramorum* may test negative for the COX internal control (no TxR Ct). In most cases the FAM Ct for *P. ramorum* is low, indicating high target DNA concentration that can inhibit the internal control amplification. One should remember that the internal control amplification is more important in cases when sample is negative for *P. ramorum* since in this case a positive COX reaction indicates that sample is negative for *P. ramorum* because of the absence of the pathogen target not because of bad quality DNA or presence of inhibitors. To resolve the problem two courses of action can be followed: 1) Dilute the DNA further (1:1,000 to 1:10,000) and repeat the real-time PCR; however, this may not work since inhibition sometimes depends on the targets ratio and not absolute concentration. If the retest again produces no fluorescence (COX internal control Ct = 0-13) or very high fluorescence (Ct = 38-46) then the sample may have to be re-extracted and retested; or 2) Rerun a 1:10 and 1:100 dilution of the sample in the *P. ramorum* conventional Multiplex PCR assay. The internal control amplification in the conventional Multiplex PCR is less likely to be inhibited by high *P. ramorum* concentration and the NS1+2 internal control primers amplify not only plant DNA but *Phytophthora* spp. DNA.



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### **C.3. Determining a *P. ramorum* positive sample:**

A sample is determined to test positive for *P. ramorum* if it produces a FAM Ct value between 14 and 36.00 and the controls (both those for the run and for the COX internal controls for individual reactions) gave the expected results, and no Ct was present in the negative control reaction.

Samples which test positive for *P. ramorum* should be retested by qPCR to confirm the first run results.

Samples that have FAM Cts from 36-46 should be diluted at 1:100 and retested. If the FAM Ct values are again 36-46 then the DNA sample must be subjected to evaluation using the validated *P. ramorum* conventional nested PCR.

***If there are doubts about any sample with Cts between 36 and 46, or about results from the follow-up nested PCR evaluation, the DNA sample can either be re-extracted and re-tested, or be forwarded to an approved laboratory for further analysis. Note: If this is a Potentially Actionable Suspect (PAS) sample in need of federal confirmation, see submission instructions below.***

### **D. Samples for confirmation at NPGBL:**

A **Potentially Actionable Suspect (PAS) sample** is a sample from a potential new location, a putative new host, a previously certified nursery, a geographically distinct/unique environmental find, or other non-routine situation. **A PAS sample must be forwarded to NPGBL for confirmation** (see submission information below).

Any sample that is submitted to NPGBL (overnight delivery) must be accompanied by (1) a PPQ Form 391 (Sample Submission Form), (2) labeled gel photos and/or Ct values for each run (see example table below).

**Please notify** Dr. Levy prior to sending material to NPGBL by e-mail (laurene.levy@aphis.usda.gov and renee.m.devries@aphis.usda.gov) with the tracking number and estimated delivery date using the shipping address below:

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BARC-East, Bldg 580  
Powder Mill Rd  
Beltsville, MD 20705,  
(Phone 301-504-7100 and fax 301-504-8539)

**Example of Table of qPCR data to accompany the PPQ Form 391 and sample submitted to NPGBL**

Sample ID	PCR Run #	Dilution	P. ram FAM Ct Value	COX TxR Ct Value
SOD-MD-0113	1	Und	36.5	24.0
	1	1:10	36.9	26.8
	2	1:100	37.9	28.5

**Definitions:**

**Environmental Sample:** DNA isolated through a nucleic extraction process from plant material.

**Genomic DNA:** DNA isolated through a nucleic extraction process from pure *Phytophthora ramorum* culture.

**Potentially Actionable Suspect (PAS) sample:** a PAS sample is a sample from a new location, from a putative new host, previously certified nursery, unique environmental find, or other non-routine situation.

**References:**

1. K.J.D. Hughes, Central Science Laboratory, SOP No. PLHB/M28: Diagnosis of *Phytophthora ramorum* by TaqMan PCR. Version dated 09-28-2003.
2. J. Tomlinson, Central Science Laboratory, SOP No. PLHB/M31: Use of the Cepheid Smart Cyclyer II for on-site detection of *Phytophthora ramorum*. Version dated 04-23-2004.
3. SmartCycler® II Operator Manual Copyright 1999-2002 by Cepheid.

**Document Revision History**

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Status (Original/Revision/ Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	06/20/2005	Quantitative real-time PCR qPCR for <i>P. ramorum</i>
Revision	1	10/09/2005	Added additional language to the introduction, added further explanation to the test evaluation. Added an example of a data table that must accompany PAS samples for testing at NPGBL. Added definitions and references.
Revision	2	10/24/2005	Added additional information to the introduction regarding the ring-test data, included the chemical and physical parameters of the ABI 7900 and 7000 instruments as a result of the ring-test. Data in ring-test regarding BioGene Hot Taq that reduced cross reactions in the Cepheid system were not added to the WI needing further investigation and because the enzyme is not available in the US. Added definition of the PAS sample. Reformatted.
Revision	3	2/14/2006	Added comment to section A2 that Omnimix beads are not to be used with this protocol. Removed reference to WI-B-T-0-5 and WI-B-T-0-6 in section B2. Corrected Table 1 for dNTPs to read 10 mM each. Corrected Table 2 to remove '# of beads' from volume column heading. Clarified PAS in sections C2 and C3. Changed 'should' to 'must' in section D and reworded definition.
Revision	4	4/03/2006	Added language to clarify resolution of non-conforming results for the internal control (COX) assay (page 8, C.2)

**Approved: Signature on File with Original Document**

Approved By: Renee M. DeVries, NPGBL Quality Manager  
Date: 04/03/2006  
Signature:

Approved By: Laurene Levy, NPGBL Technical Manager  
Date: 04/03/2006  
Signature: